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TEXAS UNIV MEDICAL BRANCH AT GALVESTON
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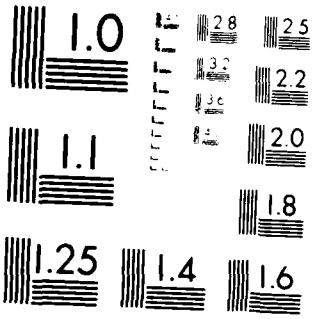

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A portion of this work has been submitted for publication in Analytical Biochemistry.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

We have proposed that an early event in wound healing involves the synthesis of hyaluronic acid (HA) by blood cells in the clot and that HA specifically interacts with fibrin to create a second matrix within the wound. Peripheral cells then migrate into the wound and begin to remodel the matrix. To test this model we have synthesized a unique derivative of HA, hyaluronate-amine, which has been used to prepare (1) radioiodinated

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20. ABSTRACT (continued)

HA to study cell surface receptors, (~~ii~~) synthetic culture surfaces to study cell interactions with immobilized HA and (~~iii~~) HA-sepharose, an affinity chromatography media. We have also used the HA-sepharose to show, as predicted by our proposed model, that fibrinogen specifically binds to HA. This is the first demonstration of such an interaction and would be of major significance in understanding the important early events necessary for prompt and efficient wound healing.

PROGRESS REPORT

a. Publications

The following publications, which have been submitted or are in preparation, have been supported by this Contract during the last 1 1/2 years.

1) Raja, R., LeBoeuf, R.D., Stone, G.W. and Weigel, P.H. (1983) Synthesis of alkylamine and ^{125}I-radiolabelled derivatives of hyaluronic acid uniquely modified at the reducing end. (submitted to Analytical Biochemistry).

2) Raja, R. and Weigel, P. (1983) Covalent immobilization of hyaluronic acid oligosaccharides, proteins and amino-molecules on synthetic culture surfaces. J. Cell Biol., a. (in press).

3) LeBoeuf, R.D., Raja, R., Fuller, G. and Weigel, P. The specific interaction between hyaluronic acid and fibrinogen. (in preparation, to be submitted to J. Biol. Chem.).

4) Raja, R., Grissom, M. and Weigel, P. Preparation of synthetic culture surfaces: A general system for the covalent immobilization of proteins, oligosaccharides and amine-containing molecules on a non-ionic matrix. (in preparation, to be submitted to Analytical Biochemistry)

A brief summary of our results and progress toward the initial goals of the contract is presented below.

b. Synthesis of ^{125}I-Hyaluronic Acid Oligosaccharides

In order to detect and quantitate specific interactions of serum or cell proteins with HA, we have taken the time to synthesize a uniquely modified derivative; HA-amine. The details are presented in the enclosed manuscript (Appendix 1). The significance of this derivative, which has not been made before, is twofold. (i) The HA-amine derivative can be converted to a hydroxyphenyl derivative and then radiolabelled with ^{125}I at a unique position in the molecule. This HA derivative has a specific activity 10-1000 times greater than anything previously available and, (ii) The HA-amine oligosaccharides can be coupled to CNBr-activated Sepharose to make an affinity chromatography media. This is the first HA-affinity support in which the oligosaccharide is covalently attached to the matrix by a single bond at a known position (in this case, the reducing end). This method preserves the native structure of HA. We believe that the development of the HA-amine derivative will prove extremely valuable to us and other investigators in this area.

c. Development of Type I and Type II Fibrin Matrices

In our original proposal we defined Type I fibrin-based matrices as being made from pure fibrinogen and Type II matrices from cell-free plasma. Two

goals of the proposal were to study the binding of HA to these matrices and to assess their ability to stimulate the synthesis of HA by various blood cells trapped within the matrix. For these purposes we did a series of studies on the physical properties and stability of Type I and II matrices. We have defined conditions (e.g. the fibrinogen concentration range) for the preparation, washing and handling of matrices in tubes and in 35 and 60 mm culture dished. Preliminary experiments with ^{125}I-HA and Type I and II matrices suggested that HA and fibrin interact in a specific and high affinity manner. These experiments were technically difficult to perform and evaluate although the results were consistent with our initial working hypothesis that HA and fibrin interact. Both of these molecules are very large, potentially mutivalent and have the ability to form gels. Consequently, the HA-fibrin interaction is not as easy to study as, for example, the interaction between a soluble univalent ligand and a protein. We therefore decided to simplify the system and evaluate other ways to study HA-fibrinogen interactions (see below).

d. Cell Interactions with Type I and II Fibrin Matrices

Conditions have been determined for culturing cells with Type II fibrin matrices formed in 35 mm culture dishes. In these experiments, human foreskin fibroblasts were used as a model cell type. Cells cultured on these matrices attached and looked healthy. Cells were also grown on tissue culture plastic and the matrix was formed on top of them or they were present during formation of the matrix and were suspended in it. In all cases, cells were viable and could be maintained in culture. Technically therefore we can do the proposed studies with blood cells to assess the effect of the extracellular matrix on HA synthesis. The assay for quantitating HA synthesis has also been adapted. These matrices can also be used to examine the ability of cells, principally fibroblasts, to migrate into and within the matrix (clot) and to screen for factors which affect this critically important process.

e. ^{125}I-HA Binding to Blood Cells

The high specific activity ^{125}I-HA derivative can be used to identify cells containing specific receptors for HA. In preliminary experiments, platelets isolated from human plasma did not show any specific binding with the ^{125}I-HA oligosaccharides. We have not yet examined HA binding to other blood cell types.

f. Synthesis of Cell Culture Surfaces Containing Immobilized HA

Synthesis of the unique HA-amine, described above, has allowed us to make flat cell culture surfaces with this extracellular matrix molecule covalently attached. This system is based on polyacrylamide chemistry and allows one to control the chemical nature of the matrix and the concentration of the immobilized ligand (11). This work, supported in part by this Navy Contract, is described in publication 2 (Appendix 2) and above. These culture surfaces will be used to study cellular interactions with HA and, in particular, the role of HA in supporting cell migration.

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g. Development of Assays to Assess HA-fibrinogen Interactions

A number of possible assay systems were explored as alternatives to the use of solid fibrin matrices to examine whether HA and fibrin can interact specifically. For example, fibrinogen was adsorbed to plastic dishes or tubes and the binding of ^{125}I-HA was assessed. In this and other cases it appeared that these two molecules interact in a strong and complicated way. To approach this problem, we have chosen to develop two methods further. One (discussed next) is affinity chromatography using HA-sepharose and the other is a solution-based assay in which fibrinogen, and any associated ^{125}I-HA, is precipitated by 50% saturated ammonium sulfate. ^{125}I-HA does not precipitate whereas > 99% of the fibrinogen does. The precipitate is collected by centrifugation, washed and radioactivity is determined. The latter method shows much promise and we will continue to develop it.

h. Affinity Chromatography of Fibrinogen on HA-Sepharose

Although we observed that ^{125}I-HA bound to fibrin matrices, the presence of other proteins as well as the polymeric and insoluble state of fibrin made these experiments difficult to interpret. To overcome these difficulties, the previously described HA-amine derivative was coupled to CNBr-activated Sepharose and used as an affinity chromatography resin to study the binding between HA and fibrinogen, the soluble monomeric form of fibrin.

Initial experiments demonstrated that when a solution of fibrinogen (2.5 mg/ml) was applied to a 1 ml column of HA-Sepharose (1.3 mg HA/ml resin) an average of 1.35 mg of fibrinogen (SEM = 0.25 mg, n = 2) was retained by the column. The possibility that the observed binding was nonspecific was tested through two independent approaches. First, we examined whether HA-fibrin binding was due to ionic interactions between these two macromolecules by attempting to elute bound fibrinogen with 2 M NaCl. This treatment eluted < 5% of the bound fibrinogen indicating that HA-fibrinogen binding was not due to simple electrostatic interactions. Secondly, we tested whether fibrinogen would bind to the CNBr-activated Sepharose resin itself, since this support is positively charged and could interact with fibrinogen in a nonspecific manner. Normally, when preparing HA-Sepharose, any unreacted sites are coupled to ethanolamine. Therefore as a control we prepared ethanolamine-Sepharose and compared the binding of fibrinogen to this resin and to HA-Sepharose. The amount of fibrinogen bound to ethanolamine-Sepharose was 17% of that bound to HA-Sepharose. Thus 83% of the fibrinogen bound to HA-Sepharose was due to specific interactions between hyaluronate and fibrinogen.

The above experiments suggested that the observed binding between HA and fibrinogen was specific. We pursued this further by evaluating the affinity of a variety of other proteins for HA-Sepharose. The proteins tested had known isoelectric points above and below that of fibrinogen so that any potential binding differences due to the net charge of the protein could also be assessed. The amount of protein bound for any of the proteins tested was much less than the amount of fibrinogen bound (Fig. 1).

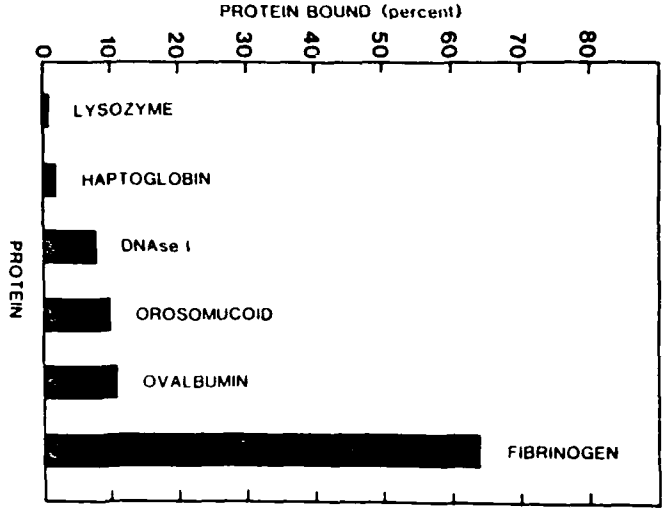


Figure 1 Binding of various proteins to HA-Sepharose. A 1 ml Sepharose column containing 1.3 mg of HA was equilibrated with 150 mM NaCl, 7 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM HEPES, pH 7.4 at 22°C. The column was treated with BSA (2 ml, 10 mg/ml) and washed until the A_{280} returned to zero. The following proteins (the pI is given in parentheses) were sequentially applied to the column and eluted with buffer: lysozyme (10.5), haptoglobin (4.0), DNase 1 (4.7), orosomucoid (2.7), ovalbumin (4.6), and fibrinogen (5.5). Three mg of each protein were applied to the column and the percentage of protein bound was determined from the A_{280} units recovered in the eluant compared to the A_{280} units applied. The column was washed extensively between sample applications until the A_{280} returned to baseline.

The specificity and reversibility of fibrinogen binding to HA was also assessed in competition trials using carbohydrate polymers with charge densities and structures similar to HA. HA, which was the most effective competitor for fibrinogen bound to HA-Separose either in the native state or as oligosaccharides, eluted a total of 49% of the bound fibrinogen from the column (Fig. 2). Chondroitin sulfate and polygalacturonic acid each eluted less than 4% of the bound fibrinogen. The next most effective competitor was dextran sulfate, which eluted about 11% of the bound fibrinogen. The amount of bound fibrinogen displaced from HA-Sepharose increased with increasing HA molecular weight (Fig. 2) indicating that the affinity of fibrinogen for HA varied with size. HA oligosaccharides were only 42% as effective as native HA in eluting fibrinogen bound to HA-Sepharose. The results of these experiments revealed that the fibrinogen binding domain for HA may be large and that long native HA polymers may be capable of multivalent interactions with fibrinogen.

The results of these reciprocal binding and competition experiments with different protein and carbohydrate species conclusively demonstrates that there is a specific high affinity and reversible binding between HA and fibrinogen. To our knowledge this is the first demonstration of a specific interaction between these two macromolecules and validates the working hypothesis of our initial research proposal. The interactions between these two molecules in vivo could provide the mechanism by which the early wound matrix is stabilized and organized.

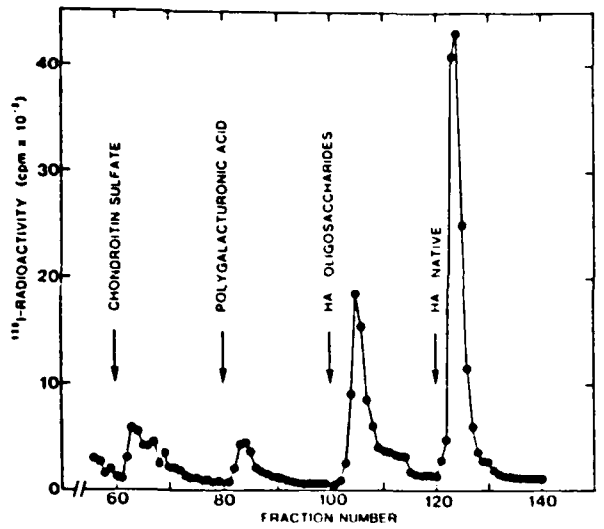
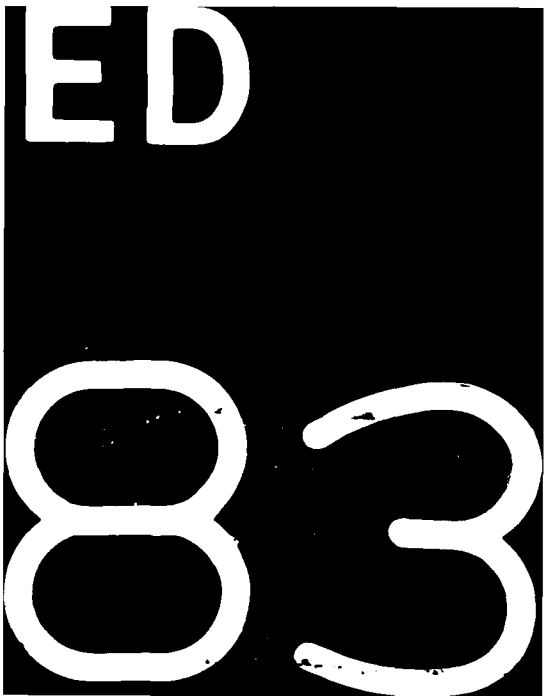


Figure 1. Release of bound fibrinogen from HA-Sepharose. Four hundred µg of ^{125}I-fibrinogen was applied to a 1 ml HA-Sepharose column (1.3 mg HA/ml resin) equilibrated with PBS. The unbound fibrinogen was washed from the column with PBS until the radioactivity per fraction (0.5 ml) returned to baseline. At the points shown by the arrows 3 mg/ml solutions of the indicated carbohydrates were applied to the column and the radioactivity per fraction was determined.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